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(54) Primers for replication and/or amplification reactions

(57) (i) Blocking primers are disclosed which may be used to prevent misannealing of specific primers to template sequences with which they are not exactly complementary. In addition, blocking primers may be employed to prevent annealing of oligonucleotide sequences produced in template repair processes from themselves acting as primers. The blocking primers may be of random sequence or of specific known sequence. A ddNTP at the 3' end of the random or specific blocking primers prevents extension reactions.

(ii) Extension reactions are disclosed which are performed using two primers for each nucleic acid strand. The primer closest to the 5' end of the target sequence may be extended whereas the primer closest to the 3' end of the target sequence (specific enhancing primer) has a ddNTP at its 3' end and thus cannot act as an initiator of nucleic acid extension. The method produces extension products of known size.

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A METHOD FOR IMPROVING REACTION STRINGENCY WHEN REPLICATING AND/OR AMPLIFYING A NUCLEIC ACID SEQUENCE

The invention relates to a method for reducing non-specific priming when replicating and/or amplifying a specific nucleic acid sequence, which enhances specificity of nucleic acid products formed during these processes.

A number of methods are now known for the amplification and replication of nucleic acid sequences. Amplification reactions include the polymerase chain reaction (PCR) for amplifying DNA sequences, reverse transcription-PCR (RT-PCR) for amplifying RNA sequences, the self-sustained sequence replication (3SR) for amplifying RNA and DNA sequences and the ligase chain reaction (LCR) to amplify DNA as well as to discriminate a single base mutation. Reviews of PCR are provided by Mullis (1986) Cold Spring Harbor Symp. Quant. Biol. 51: 263-273; Saiki *et al* (1985) Bio/Technology 3: 1008-1012; and Mullis *et al* (1987) Meth. Enzymol. 155: 335-350. RT-PCR is described by Kawasaki (1990) Amplification of RNA, in Innis *et al* Eds, PCR Protocols: A Guide To Methods and Applications, New York, Academic Press; 21-27. RT-PCR using a thermostable recombinant thermus thermophilus (rTth) DNA polymerase is described in Wang *et al* (1989) Proc. Natl. Acad. Sci. USA 86: 9719-9721, and the Perkin-Elmer Biotechnology Products Catalogue, Branchburg, New Jersey, USA (1993) P.25. 3SR is described by Gingeras *et al* (1991) Journal of Infectious Diseases 164: 1066-1074 and Gingeras *et al* (1993) Hepatology 17: 344-346. The ligase chain reaction is described in Barany F, PCR Methods and Applications, Cold Harbor Spring Laboratory Press (1991); 1: 5-16.

Replication reactions include reverse transcription which converts RNA sequences into cDNA sequences, and the primed *in situ* extension (PRINS) reaction which replicates sequences of DNA by an extension method. Methods of reverse transcription are well known, and the PRINS reaction is described in Koch J et al (1989), Chromosoma 98: 259-265.

In situ hybridisation (ISH) is a method for detecting a specific nucleic acid sequence within cells (Nagai *et al* (1987) Intl. J. Gyn. Path. 6: 366-379). The technique involves allowing a labelled probe to hybridise to the sequence of interest within the cells and then visualising the probe, usually by a microscopic technique. Labelling is by

immobilised nonisotopic (fluorescent or absorbance) or isotopic (autoradiographic) signal attached to the probe. A problem with ISH is that it is not generally highly sensitive, often requiring tens to hundreds of copies of the target sequences per cell for adequate visualisation. Hybridisation reactions using nucleotide sequence specific probes may also be performed extracellularly. Recently, techniques have been developed which allow PCR to be performed intracellularly ("*in situ* PCR") with or without prior reverse transcription intracellularly ("*in situ* RT"). The amplified nucleic acid can be detected intracellularly by ISH, or by using a labelled primer or a labelled deoxyribonucleoside triphosphate (dNTP). The components for reaction can enter the cells, but longer amplificants cannot leave and are therefore retained in the cells for detection. This facilitates the detection of low copy number sequences (Nuovo GJ. PCR *in situ* Hybridisation Protocols and Applications. Raven Press, New York, pp 157-255). We have filed applications concerning the performance of intracellular nucleic acid reactions on a surface (PCT-GB 19061) (1993) which can be used to evaluate large sections and do not require vapour barriers or hot start techniques. Detrimental evaporation effect is similarly less critical with our protocol.

The target nucleic acid sequence may be a DNA or RNA sequence for amplification reactions. When the target sequence is a DNA sequence PCR, 3SR, ligase chain reaction or another DNA amplification reaction may be used. When the target sequence is an RNA sequence RT-PCR, 3SR or another RNA amplification reaction may be used.

These amplification reactions may have certain features in common. The reactions generally comprise carrying out multiple cycles of:

(A) hybridising (annealing) a first primer to a site in a nucleic acid strand at one end of the target nucleic acid sequence, and hybridising a second primer to a site corresponding to the opposite end of the target sequence in the complementary nucleic acid strand;

(B) synthesising (extending) a nucleic acid sequence from each respective primer; and

(C) denaturing the double stranded nucleic acid produced in step (B) so as to form single stranded nucleic acid.

The amplification mixture contains the reagents necessary for sequence

amplification. The mixture generally comprises:

(AA) a first primer which hybridises to a site in a nucleic acid strand at one end of the target sequence, and a second primer which hybridises to a site corresponding to the opposite end of the target sequence in the complementary nucleic acid strand;

(BB) an enzyme which synthesises nucleic acid; and

(CC) at least four dNTPs and/or at least four NTPs.

The mixture also generally contains an aqueous buffer and a water soluble magnesium salt.

The target sequence may be a DNA or RNA sequence for replication reactions. When the target sequence is a DNA sequence, a PRINS reaction or another replication reaction may be used. When the target sequence is an RNA sequence, reverse transcription or another replication reaction may be used.

These replication reactions may have certain features in common. The reactions generally comprise carrying out a single cycle or multiple cycles of:

(1) hybridising (annealing) a primer (or multiple primers) to one or more sites in a nucleic acid strand.

(2) synthesising (extending) a nucleic acid sequence.

For a DNA target, an initial denaturation step to separate the nucleic acid strands is helpful. It is also possible to perform multiple cycles of replication reactions.

The replication mixture contains the reagents necessary for sequence replication. The mixture generally comprises:

(11) A primer or multiple primers which hybridise to one or more sites in a nucleic acid strand

(22) An enzyme which synthesises nucleic acid; and

(33) At least four dNTPs and/or at least four NTPs.

In situ hybridisation generally includes the pretreatment of biological material to increase permeabilisation of cell membranes, and the labelling of a nucleic acid sequence to form a probe. Labelling a nucleic acid involves the incorporation of either a radioactive or nonradioactive marker which can be detected. A denaturation step is of value with a DNA target.

A primer is an oligonucleotide which hybridises to a nucleic acid sequence and

directs initiation of synthesis of the complementary strand. A primer is usually from 10 to 100 nucleotides in length, for example from 12 to 60 nucleotides in length.

The enzyme which synthesises nucleic acid in an amplification or replication reaction could be a DNA or RNA polymerase. The enzyme is frequently thermostable, since it should generally be able to withstand the temperatures of the amplification or replication reaction which are often elevated (eg from 30 to 100°C). Self-sustained sequence replication (3SR) and reverse transcription generally require lower temperatures (eg from 20°C to 45°C).

The dNTPs and NTPs which may be used include (deoxy)guanosine triphosphate, (d)GTP; (deoxy)cytidine triphosphate, (d)CTP; (deoxy)thymidine triphosphate, (d)TTP; and (deoxy)adenosine triphosphate, (d) ATP. However,other dNTPs and NTPs may be used, for example dUTP and UTP.

PCR comprises carrying out cycles of the following steps:

(C') denaturing double stranded DNA containing the target sequence to obtain single stranded DNA;

(A') hybridising (annealing) a first primer to a site in a DNA strand at one end of the target sequence, and hybridising a second primer to a site in the complementary strand corresponding to the opposite end of the target sequence; and

(B') synthesising (extending) DNA from the first and second primers.

The number of cycles may be up to 40 or 50, for example from 25 to 40, preferably from 30 to 40. Cycling through the denaturation step, hybridisation step and synthesis step is effected by cycling the temperature of the reaction mixture ("thermal cycling"). Denaturation is generally carried out at from 80 to 100°C, hybridisation (annealing) is generally carried out at from 40 to 80°C, and extension is generally carried out at from 50 to 80°C. A typical cycle is denaturation: about 94°C for about 1 min, hybridisation: about 58°C for about 2 min, and extension: about 72°C for about 1½ min with five seconds added to the extension time on each consecutive cycle. The exact protocol depends on factors such as the length and sequence of the primers and target sequence, and the enzyme used.

The amplification mixture for PCR contains all the reagents necessary to effect amplification by PCR. These reagents include the following:

a first primer which hybridises (anneals) to a site in a DNA strand at one end of the target sequence and a second primer which hybridises (anneals) to a site in the complementary strand corresponding to the opposite end of the target sequence; a thermostable DNA polymerase (ie a DNA polymerase which does not lose significant activity at the temperatures used in thermal cycling, for example up to 95°C); and at least four dNTPs. An aqueous buffer and a water soluble magnesium salt are also generally required.

It is also possible to use multiple overlapping primers to produce large amplificants, but this is not usually necessary.

RT-PCR is a modified form of PCR for amplifying RNA sequences. The target RNA sequence is first converted to a complementary DNA (cDNA) sequence and the cDNA is then amplified.

RT-PCR may be carried out using a single enzyme which performs both the RT and PCR reactions (eg thermostable recombinant *Thermus thermophilius* (rTth) DNA polymerase, Perkin-Elmer number N 808-0069). Alternatively, RT-PCR may be performed using a different enzyme for each of the reactions, for example reverse transcriptase for the RT reaction and DNA polymerase for the PCR.

The main advantage of the rTth DNA polymerase over using two different enzymes is that reverse transcription can be carried out at a higher temperature thereby permitting amplification of the sequence of an RNA molecule with a complex secondary structure. rTth DNA polymerase reverse transcribes RNA to cDNA in the presence of Mn^{2+} ions, and acts as a DNA polymerase after chelation of the Mn^{2+} ions. rTth DNA polymerase may be used in accordance with the manufacturer's instructions (Perkin-Elmer catalogue number N 808-0069) except that the reaction mixture should preferably be diluted so as to be isotonic for optimal morphological preservation. The reaction mixture for RT by rTth DNA polymerase generally comprises a pair of primers as described above; the rTth polymerase; at least four dNTPs; and a water soluble manganese salt. The Mn^{2+} ions are chelated after RT with a chelating agent in order to begin a PCR. The reaction mixture also generally contains an aqueous buffer and a water soluble magnesium salt.

When two different enzymes are used to perform the two steps for RT-PCR, the first RT step involves a replication reaction synthesising DNA from RNA using reverse

transcriptase. The reagents for this generally comprise a "downstream" primer or random hexamer, reverse transcriptase, at least four dNTPs, a buffer, a water soluble magnesium salt, and an RNase inhibitor. Only a single primer is required for RT, but is also possible to use a mixture of primers of random sequences (eg random hexamers). A single primer may have the sequence of the 5'-end of the target sequence or be an oligo dT primer (poly (rA) tail primer).

3SR is an RNA amplification system which relies on the combined activity of three enzymes: reverse transcriptase (eg avian myeloblastosis virus (AMV) reverse transcriptase), RNA polymerase (eg T7 RNA polymerase), and a ribonuclease which digests RNA in DNA:RNA duplexes (eg ribonuclease H (RNase H)). The principles and methods of 3SR are clearly explained by Gingeras *et al* (1993) Hepatology 17: 344-346. After denaturing the RNA at elevated temperature (eg at from 40 to 100°C), reagents including the following are added and incubated at from 30 to 50°C (preferably 37 to 42°C) for from 15 min to 5 h (preferably from 1 to 2 h):

a first primer which hybridises to a site at the 3'-end of the target sequence and a second primer which hybridises to a site complementary to the 5'-end of the target sequence, at least the first primer having an initiation sequence for RNA polymerase in addition to the sequence which hybridises to the target sequence; a reverse transcriptase, an RNA polymerase and an RNase which degrades RNA in DNA:RNA duplexes; and at least four dNTPs and at least four NTPs. It is also generally necessary to use an aqueous buffer and a water soluble magnesium salt.

The basic 3SR technique can be modified to amplify DNA sequences. To do this, a reaction mixture without enzymes is heated at from 80 to 100°C to denature the double stranded DNA. The temperature is then reduced, ranging from 30 to 70°C (eg 42°C) to allow primer hybridisation. Reverse transcriptase is then added for from 2 min to 1 hr (eg 10 min) before a second denaturation and hybridisation step. All three enzymes are then added, and the reaction proceeds as normal.

3SR has certain advantages over PCR, the greatest of which is ease of use. No thermal cycling is necessarily required, and it may simply be necessary to perform the initial denaturation step, add all the reagents and then incubate them with the sample. 3SR is therefore well-suited to screening large numbers of samples for RNA or DNA sequences of interest. The allele-specific ligase chain reaction uses four

oligonucleotides, two adjacent oligonucleotides which uniquely hybridise to one strand of target DNA, and a complimentary set of adjacent oligonucleotides which hybridise to the opposite strand. Thermostable DNA ligase will covalently link each set as long as complete complementarity exists at the junction. Since oligonucleotide products of one round may act as substrates for the next round, the signal is amplified. A single base mis-match at the oligonucleotide junction will not be amplified and will therefore be distinguished. Components for performing this reaction are described in Barany F (1991): PCR Methods and Applications 1: 5-16. Reaction components may be diluted to produce an iso-osmotic environment for cells.

The primed *in situ* extension (PRINS) reaction uses one or more oligonucleotides which uniquely hybridise to one template strand of RNA or denatured DNA. A polymerase or replicating enzyme, for example, Taq polymerase or Reverse Transcriptase, will then promote extension of a new nucleic acid sequence complimentary to the original template strand. The process may be repeated to perform an amplification reaction (PCR-PRINS) by repeated replication processes.

The amplification or replication mixture used in accordance with the invention may be iso-osmotic with the cells (isotonic for human cells) and contain the reagents necessary for amplification or replication of the target nucleic acid sequence. The mixture is essentially conventional, except for the fact that it is iso-osmotic with the cells (and therefore diluted). Dilution may be carried out with water (preferably distilled water) until the mixture is iso-osmotic. The volume of mixture may be increased further by adding an iso-osmotic diluent (eg phosphate buffered saline (PBS)). For human cells the osmolarity of the mixture may be from 2.75 to 29500 mOsmol/kg, but optimal results may be obtained when the mixture is approximately isotonic, for example, from 100 to 500 mOsmol/kg.

The amount of Taq polymerase in a PCR mixture per sample may be from 0.1 to 3000 units, preferably 1 to 30 units, for example, about 5 units. The amount of reverse transcriptase in an RT mixture (per sample) may be from 2.5 to 3000, preferably 25 to 400 units, for example, about 250 units. The amount of RNase inhibitor in an RT mixture (per sample) may be from 1 to 3000 units, preferably from 10 to 300 units, for example, about 100 units. The amount of magnesium ions and Taq polymerase do not have to be disproportionately increased, but can be maintained in proportion to the other

components used in conventional PCR. Thus, all components can be diluted equally.

For either extracted nucleic acids from cells, or for cells in suspension, the PCR reaction may be performed in a tube after addition of a PCR mixture. Alternatively, the test sample may include a surface on which cells are maintained, or a container on a surface in which cells or liberated target material are kept in solution including components for intracellular chemical reaction. The said surface on which cells are maintained may be covered with a reaction mixture contained within a well formed by a biocompatible semi-adhesive filler which surrounds the cellular sample; the well is then covered by a coverslip, for example in a similar manner to that previously described (PCT GB9406841.8). In contrast, the said surface on which cells are maintained may be covered by a reaction mixture enclosed in a preformed reaction container which is specifically designed to surround the cells on a surface, for example as described in a previous patent application (UK.9402205.0).

Detection of the target nucleic acid sequence may be carried out using one of two strategies. The first strategy is to label a primer, a dNTP or an NTP in the amplification mix, so that the amplified nucleic acid product is labelled and can be detected directly. The second strategy is to carry out ISH employing a labelled probe which hybridises to a part (eg at least 10 nucleotides) or the whole of the target sequence. ISH is a well-known technique which involves the steps of hybridising a labelled probe to the target sequence; and washing away non-hybridised probe. The hybridised probe is then detected. For DNA sequences, an initial denaturation step may be required.

A probe is a labelled nucleic acid (DNA or RNA) which hybridises to a part or the whole of the target sequence or the sequence complementary to the target sequence. A probe is generally from 10 to 5000 nucleotides in length, for example from 12 to 100 nucleotides in length.

A primer, a probe, a dNTP and an NTP can be labelled in a number of different ways, for example with a radioactive label (eg ^{35}S, or ^{32}P), biotin (which may be detected using avidin or streptavidin conjugated to peroxidase), a fluorescent label (eg fluorescein), or digoxigenin. A preferred labelled dNTP is digoxigenin-labelled dUTP (Dig-11-dUTP). Methods of detecting these labels are well-known. Detection can be quantitative (eg image analysis, gel electrophoresis, grain counting of a radioactive label or flow cytometry) or non-quantitative (eg histological examination).

It is also possible to carry out gel electrophoresis optionally followed by Southern blotting (for DNA) or Northern blotting (for RNA) of the nucleic acid produced in the reaction. Indeed, this is an important control for checking that the nucleic acid which is produced is the target nucleic acid. Gel electrophoresis can be used to check that a nucleic acid of the expected size is produced. The identify of the nucleic acid can be confirmed by Southern (DNA) or Northern blotting (RNA) using a probe. Southern and Northern blotting are well-known techniques which comprise transferring nucleic acids separated by gel electrophoresis onto a solid support (eg a nitrocellulose support) by blotting, and hybridising a probe to only target nucleic acid.

The nucleic acid within the cells may be prepared for gel electrophoresis by release from the cells by lysis, centrifugation to a pellet together with the cell debris, and resuspension of either supernatant or residue or both in PBS and in gel loading buffer. Gel electrophoresis may also be carried out on DNA extracted from cells and/or on unevaporated amplification mixture and/or cell lysates on a surface. Further, the nucleic acid resulting from the reaction can be sequenced by a conventional sequencing method.

The cells containing the target nucleic acid sequence are generally eukaryotic cells, generally animal or human cells, though plant or prokaryotic cells can be used. The cells may be in a tissue section (ie a solid sample of biological tissue), a cytospin (ie a cell suspension which has been spun by centrifugation onto the first surface), a biopsy (eg a needle biopsy, a mucosal biopsy, a mucosal curetting or a bone biopsy), a touch preparation, or other cytological specimen (eg exfoliate material, a smear, an aspiration or fine needle material). The method of the invention could also be applied to liposomes or enclosed vesicles containing nucleic acid. Alternatively, the nucleic acid (either RNA or DNA) may be directly extracted from the cellular material. The methods for extraction are well known.

The cells may be fixed or unfixed. Unfixed cells may be particularly suitable for studying virus infected cells. Fixing may be carried out by any chemical or physical treatment which strengthens and preserves cellular structures, including membranes, against disruption by physical or chemical forces. Chemical fixatives which could be used include Zenkers, glutaraldehyde, Bouin's, Picric-acid based fixatives, alcohol, alcohol-based fixatives, 10% buffered formalin, paraformaldehyde, mild detergents and osmium tetroxide. Physical fixation methods which could be used include freezing and

microwaving. Tissue sections will usually be sliced into thin sheets (typically, several microns thick). Tissue sections can also be embedded in a wax (eg paraffin) or a plastic, which is removed (eg by washing with xylene). Tissue sections can also be cut from a frozen sample of biological tissue to produce a frozen tissue section. No DNase or RNase pre-treatment is required, although they can be carried out. The cells may be treated so as to assist entry of the amplification, transcription or ISH reagents, for example, with proteinase K or trypsin.

A major consideration in amplification and replication reactions is mispriming. Mispriming refers to the hybridisation of one or more oligonucleotide sequences to a region of a nucleic acid strand which does not have a unique complimentary sequence, and initiation of an extension reaction, since one or more of the nucleotides are mismatched, for example, thymine-thymine rather than thymine-adenine. The subsequent amplification or replication reaction would therefore not be specific. The consequence of this nonspecific reaction would generally be a false positive signal after detection. In addition, if both a specific and a nonspecific reaction are occurring simultaneously, the nonspecific reaction would compete for reagents with the specific reaction, resulting in a much weaker specific reaction.

Mispriming of sequences to a non-complimentary region of the nucleic acid template tend to occur with less stringent reaction conditions compared with specific hybridisation of a primer to an exact complimentary region of a nucleic acid strand. A number of strategies have been used to increase stringency of reaction conditions irrespective of any particular specific nucleotide sequence of interest. "Hot start" refers to withholding a vital component of a PCR reaction mixture until a suitable hybridisation (annealing) temperature is reached where theoretically only specific hybridisation can occur. At that temperature, the vital component is added and the PCR reaction commences (Nuovo GJ. PCR *in situ* Protocols and Application (1992). Raven Press, New York). A second strategy for PCR involves maintaining relatively high hybridisation (annealing) temperatures, at least in the early stages of reaction, and then either maintaining the same hybridisation temperature for the remainder of the reaction, or progressively lowering the hybridisation temperature progressively for the remainder of the reaction (for example, so-called "Touchdown PCR"). A third strategy involves altering conditions for PCR including reaction components. It is suggested that reduction

of primer concentration, enzyme concentration, concentration of dNTPs, magnesium, or number of PCR cycles; enhance specificity (Innis et al, PCR Protocols: A Guide to Methods and Applications, New York, Academic Press). A fourth strategy for PCR involves nested reactions, in which the PCR product of one PCR cycling reaction itself becomes a template for a second PCR reaction with a second primer pair with specificity for sequences of the first round of PCR product. A number of other strategies to increase stringency of reaction conditions for amplification and/or replication reactions have been proposed, which are used without regard to any particular specific sequence of interest. A number of reaction components are used in many hybridisation (or annealing) reactions to enhance specific hybridisation of primers to target DNA. These include unlabelled random sequence blocking DNA or tRNA to prevent hybridisation of specific probe to nonspecific sites. Although these random blocking agents have definite advantages for *in situ* hybridisation, blocking DNA (or RNA) may itself become a primer hybridising to template sequences different from the specific sequence of interest, and initiate non specific amplification or replication reactions. Furthermore, the random blocking agents are clearly of little value in blocking closely homologous sequences to the target sequences using random oligonucleotides, for example in blocking closely related allelic sequences or mutations, due to the random nature of the blocking DNA. Consequently, closely homologous target sequences may have very similar hybridisation stringency conditions and discrimination by increasing stringency alone using random blocking oligonucleotides would be of little value.

To reduce mispriming of nucleic acid sequences to closely-related template regions to the nucleotide sequence of interest, it is prudent to adopt a strategy of blocking the template sites by specially constructed specific nucleic acid sequences, hereafter referred to as specific blocking oligonucleotides. In contrast, to reduce mispriming of nucleic acid sequences to any template region which is not closely related to the sequence of interest, it is of value to adopt a strategy of blocking the template sites by random nucleotide sequences, hereafter referred to as random blocking oligonucleotides. To prevent these misprimed initiation of replication or amplification reactions by either specific or random blocking oligonucleotides, it is clearly advantageous to have a dideoxynucleotide (hereafter referred to as ddNTP) present at least in the 3'-end of the oligonucleotide. The ddNTP at the 3'-end of the respective

blocking oligonucleotide would prevent misprimed initiation of transcription or amplification reactions, since ddNTPs are used routinely to cause termination of amplification in sequencing reactions, the method of which is well-known. The importance of maintaining a ddNTP at least in the 3'-end of the oligonucleotide due to the fact that generally amplification and replicating reactions proceed in a 5' to 3' direction. Since no amplification or replication reaction occurs for *in situ* hybridisation reactions, the presence of a ddNTP at the 3' end of the oligonucleotide is not necessary.

For amplification reactions, the presence of blocking oligonucleotides with ddNTP at the 3'-end, hybridise to the template sequences at low hybridisation (annealing) temperatures; and therefore compete with specific reaction primers from hybridising (annealing) to template sequences different from the specific sequence of interest. At higher hybridisation (annealing) temperatures, any blocking oligonucleotides bound to the specific sequence of interest will dissociate since they will not be exactly complimentary to the said specific sequence; specific primers will then hybridise to the specific sequence of interest. Since reactions between specific primers and their respective specific complimentary sequences on the template are stable at relatively high hybridisation temperatures, a specific reaction product occurs. In addition, hybridisation of blocking oligonucleotides to template sequences which remain hybridised at high hybridisation (annealing) temperatures similar to that for specific primer hybridisation, will not initiate replication or amplifiction reactions due to the ddNTP at the 3'-end of the respective blocking primers.

When discrimination of closely related sequences of a nucleic acid template is performed, for example in evaluating different alleles or mutations in different alleles, it is helpful to include specific blocking oligonucleotides with exact complementarity to the said closely related sequences but not for the sequence of interest. The presence of the specific blocking oligonucleotides for these closely related sequences therefore adds a further level of stringency in performing nucleic acid replications or amplification. Where discrimination between less closely related nucleic acid template sequences is performed, then random blocking oligonucleotides may be employed to block less closely related nucleic acid template sequences. A greater yield of specific amplification product will therefore be obtained. Alternatively, both specific blocking oligonucleotides and random blocking oligonucleotides may be used simultaneously or in combination.

For specific amplification or replication reactions, it is advantageous to produce specific reaction products of defined size, which then act as specific templates for further reactions. Replication and the first few cycles of amplification reactions do not produce reaction products of defined size in a predictable manner. With regard to replication or amplification reactions, leakage across cell membranes will depend on sample fixation and pretreatment times; if the reaction product size after replication reactions or first few cycles of amplification reactions is known, some prediction of amount of reaction product leakage across cell membranes may be possible. In addition, shorter replication products may reduce nonspecific adsorption of the said products onto cellular structures, for example proteins, thereby reducing "background" signal during subsequent *in situ* hybridisation reactions with a suitable labelled probe. The chances of mismatch reaction product also decreases with the length of the reaction product. With regard to replication reactions, shorter replication products are advantageous since the probability of intramolecular hairpin loop formation in the antimessenger decreases with its length and accessible regions in DNA or RNA are frequently very limited in size. With regard to some amplification reactions, for example the polymerase chain reaction (PCR) or cycling primed *in situ* extension reaction (cycling PRINS), amplified products of defined size are advantageous since template is constantly being degraded during thermal cycling; shorter templates are less likely to be degraded. It is therefore of advantage to use two primers exactly complementary to two different defined regions of each nucleic acid template strand. During reaction one primer binds to the template closer to 5' end and the second primer binds to the template closer to the 3' end; the second primer has a ddNTP bound at its 3' end preventing the said second primer from itself acting as an initiator of nucleic acid extension. During reaction, the primer closer to the 5' end will initiate an extension reaction, which will terminate when the second primer closer to the 3' end is reached; no extension can occur beyond the 3' end of the second primer since a ddNTP is present at that site. The said second primer with a ddNTP at the 3' end shall hereafter be referred to as a "specific enhancing primer".

The above invention may be used alone or in combination with other agents that promote a specific replication or amplification reaction, since no single strategy described previously in the art will itself guarantee a specific replication or amplification reaction product. It is sensible therefore to combine the different strategies outlined to give the

highest possible specific reaction yield. Alternatively, specific blocking oligonucleotides would increase specificity of *in situ* hybridisation.

The method of the invention is useful for detecting nucleic acid sequences within cells, particularly low copy number sequences. Low copy number bacterial (eg mycobacterium) or virus infection (eg DNA viruses or RNA viruses can be detected. In addition, by evaluating proviral genetic sequences or ratio of positive and negative strands of virus, a measure of viral replication can be made. With regard to tumours; mutations, specific deletions, translocations or even degrees of amplification can be documented. Detection of the percentage of cells or subpopulations showing progressive deletions (eg in colon cancer) may be of prognostic value. In lymphomas, similar methods may be used for diagnostic work and to monitor progression of one lymphoma type to another.

Evaluation of gene pleomorphism is another use of the invention at a cellular level. The invention would be particularly useful for evaluating risk factors in genetic disease, as well as for forensics, paternity testing and sex determination. By comparing the results obtained using an RNA amplification technique and those obtained using a DNA amplification technique, the ratio of mRNA to DNA for a particular gene could be obtained. This would be of value for evaluation of the degree of growth factor, oncogene or antioncogene expression in tumours, or for microbial replication. A cell subpopulation expressing a growth factor or infected with a microorganism could be identified. Normal tissue histogenesis, animal biology and pathology, microbiology, plant biology and pathology, cell biology and embryogenesis may be investigated. In endocrinology, normal and pathological hormonal expression can be studied. For research purposes, sequence tagged sites and "libraries" of primer pairs could be made. The technology might also be used for morphologically poorly preserved cells, or for cases wherein sufficient cellular material is present for routine diagnosis. Finally, the invention could be used in gene therapy.

Thus, the method of the invention has value not only in diagnosis but also prognosis of routine histological material, as well as in research. However, it is mandatory that the results obtained are specific, otherwise erroneous conclusions in research, or inappropriate diagnosis and clinical management will be made. The present

invention adds a valuable level of stringency to produce specific results suitable for interpretation.

The following Examples illustrate the invention.

EXAMPLES

Example 1

MATERIALS AND METHODS FOR *IN SITU* PCR AND DETECTION OF CYTOMEGALOVIRUS (CMV) (DNA VIRUS) IN CELLS AND TISSUES

Formalin-fixed, paraffin-embedded CMV-infected MRC-5 fibroblast cell line, CMV-infected lung tissue and uninfected tissue were obtained from histological archives. Samples were examined using routine haematoxylin/eosin stain, and by an immunohistochemistry (CMV) antibody technique (Dako Ltd, High Wycombe, UK).

Preparation of Tissue Sections

Tissue sections on APES-glued slides were cut from blocks of formalin fixed, paraffin-embedded CMV-infected MRC-5 fibroblast cell line and lung tissue. These sections were subsequently deparaffinised by successive washes with xylene and progressively diluted alcohol solutions, and allowed to air dry before being stored at -20°C before use.

Preparation of Tissue Sections for <u>In Situ</u> PCR for CMV

Stored tissue sections were surrounded by heat-resistant gum (Cowgum Unit No. 1133, Cow Proofings Ltd, Slough, UK) and allowed to set. The flat surface (or inserted heating block) of the PCR machine (Techne PHC-2 Thermal Cycler, Techne Ltd, Cambridge, UK) was covered by a heat-resistant, rapid temperature transmitting plastic tray (Techne Hi Temp Microplate, Techne Ltd, Cambridge, UK). Subsequently, the tissue sections on slides were placed onto this tray (Figure 1). DNA was also extracted from CMV infected and uninfected MRC-5 fibroblast cell line by standard techniques, and submitted for PCR analysis.

An isotonic PCR mixture was prepared according to the following specifications (partly using the Perkin Elmer GeneAmp RNA PCR kit, Perkin-Elmer Corp, Norwalk, USA) in order to produce a final volume of approximately 419 μl/slide: 25 mM $MgCl_2$

10 μl, 10X PCR buffer 20 μl, each 10 mM dNTP 5 μl, distilled deionised water 164 μl, each CMV primer (100 mg/μl) primer 2 μl, Amplitaq (Trade Name) DNA polymerase 5 U, phosphate buffered saline 200 μl. Primer sequences used were (5'-3'):

(1) ACCACCGCACTGAGGAATGTCAG and

(2) TCAATCATGCGTTTGAAGAGGTA, producing amplificant product size of 100 base pairs (Cranage *et al* (1986) EMBO J 5: 3057-3063). The PCR mixture was placed onto the slide within the well created by the gum. A plastic coverslip cut from the heat-resistant plastic trays (Techne) was finally placed onto the gum, sealing in the PCR mixture.

Intracellular PCR (In Situ PCR)

Optimal temperature settings found previously were used: Denaturation: 94°C, 1 minute/Reannealing: 58°C, 2 minutes, Extension: 74°C, 1½ minutes with a 5 second extension added to each consecutive cycle, 40 cycles. The plastic coverslip was simply removed after PCR and the glue scraped off (Figure 2).

Gel Analysis of PCR Products

The remaining non-evaporated mix overlying tissue sections after PCR was removed. 12 μl of the non-evaporated mix was mixed with 4 μl DNA-loading dye, and electrophoresed on a 1.5-3% agarose gel in TBE buffer (Tris borate EDTA electrophoresis-buffer) with ethidium bromide (10 mg/ml). Molecular weight markers (eg PUC 19 cut with Dde T) were included with each gel, and results recorded on Polaroid (Trade Name) photographs using ultraviolet fluorescence.

In situ Hybridisation (ISH) with Biotinylated CMV DNA Probes

After removal of non-evaporated mix overlying tissue sections, tissue sections were air-dried and stored at -20°C before ISH. *In situ* hybridisation (ISH) using virus specific biotinylated probes (Cambridge Bioscience, Cambridge, UK) was performed according to the method of Gall *et al* (1971) Methods Enzymol; 38: 470-473. Known positive and negative control samples were included in each ISH. Samples were treated with Proteinase K (1 mg/ml) or left untreated. Evaluation was performed by double blinded

studies with experienced histopathologists. In addition, the numbers of positively reacting cells were counted using a chromatic Image Analysis System (Leading Edge, Science Park, Adelaide, Australia). Fields containing cells were defined interactively and the cells detected by automatic thresholding. The numbers of cells were counted automatically and the density of staining of each cell assessed also automatically on the basis of grey level measurements. A magnification of 250 times was used.

PCR on CMV Sequences at low Annealing temperatures to Evaluate Effects of ddNTP labelled Blocking Oligonucleotides

The annealing temperature was decreased to 30°C from 58°C, to investigate the presence of bands on gel electrophoresis of unexpected size indicating nonspecific PCR products. The effect of addition of random blocking oligonucleotide sequences with ddNTP at the 3'end to the previously described PCR reaction mixture was then evaluated in a subsequent experiment. ddNTP labelled oligonucleotide sequences used were at similar concentrates to the specific primers used for PCR.

RESULTS

Cytopathic Changes and Immunostaining for Cytomegalovirus

CMV-infected fibroblasts and lung exhibited cytopathic effects characteristic of CMV when examined after routine haematoxylin and eosin staining. Tissue sections of CMV-infected fibroblasts and lung immunostained for CMV were positive with satisfactory controls. Immunostaining for CMV on known positive and negative control samples gave expected results.

Specificity of PCR Primers

The primer pair for CMV was tested on cell lysates and extracted DNA of CMV-infected cultured MRC-5 fibroblasts. A single band of the expected size (100 bp) was observed on gel electrophoresis. An identical band was found after nested PCR using

outer flanking primers described (Cranage *et al* (1986) EMBO J 5: 3057-3063) and identical inner flanking primers.

Gel Electrophoresis

No definite band was identified when residual PCR mixture was removed from slides after PCR and gel electrophoresis performed, though in most cases insufficient fluid was present on the slides for this procedure.

Gel Electrophoresis of PCR Products of CMV Sequences at low Annealing temperatures to Evaluate Effects of ddNTP labelled Blocking Oligonucleotides

When low annealing temperatures (30°C) were used instead of 58°C described previously, bands of unexpected size were seen on gel electrophoresis. These bands were markedly reduced in intensity when ddNTP labelled blocking oligonucleotides were added to the PCR mixture.

***In situ* Hybridisation**

In situ hybridisation performed using commercially available double-stranded DNA probes (Cambridge Bioscience Ltd, Cambridge, UK) revealed markedly increased signal after *in situ* PCR on CMV-infected lung and fibroblast tissue sections relative to unamplified control tissue sections. This was found both by blinded study examination by experienced histopathologists and by image analysis. ISH on known positive and negative controls gave appropriate results. Negative control specimens submitted for *in situ* PCR gave negative staining by ISH. Extracted DNA on negative control tissues used for *in situ* PCR also gave no band by gel electrophoresis. The same result was obtained on three successive occasions. Of interest, unset glue tended to partially cover the tissue section; a problem overcome by allowing the glue to set firm for several minutes.

Proteinase K treatment (10 μg/ml for 25 minutes at 37°C) was apparently essential for performing ISH on CMV-infected formalin-fixed, paraffin-embedded lung and fibroblast tissue on slides, since probe apparently could not penetrate the tissue section without Proteinase K predigestion.

Of interest, staining for CMV was remarkably preserved even after all the PCR mixture had evaporated off, providing that this was limited to a few cycles. However,

after evaporation of fluid, the glue became much firmer and less easy to peel. Little difference in intensity of staining between 35 and 40 cycles was obtained.

Image Analysis Results for CMV-infected Tissues

A statistically significant difference was found between test tissue sections on slides (*in situ* PCR followed by ISH) and control slides (*in situ* PCR mixture and thermal cycling but no TAQ polymerase added followed by ISH), both for intensity of signal and for number of positive cells detected.

Example 2

MATERIALS AND METHODS FOR COMBINED *IN SITU* REVERSE TRANSCRIPTION (RT) AND *IN SITU* PCR (OR *IN SITU* NESTED PCR) FOR MEASLES (RNA VIRUS) IN CELLS AND TISSUES

Cells and Tissues

Archival brain tissue showing histological evidence of subacute sclerosing panencephalitis (SSPE) of known measles aetiology was obtained. Tissues were examined for cytopathic effect by haemotoxylin-eosin stain, and by immunohistochemistry using a monoclonal anti-measles antibody (Serolab, Crawley, Sussex). Human Vero cells were also cultured, infected with measles virus and used for experiments when 80% of cells had a prominent cytopathic effect.

Method for intracellular reverse transcription (in situ RT) in tissue sections or cytospins on slides for measles virus.

Tissue sections on PLL- or APES-coated slides for investigation were deparaffinised under RNase free conditions, after being cut from formalin-fixed, paraffin-embedded tissue blocks. Deparaffinisation (RNase-free conditions) consisted of successive washes in xylene and progressively diluted alcohol conditions, before air-drying and storing at -20°C. In addition, proteinase K or (10 μg/ml 25 minutes 37°C) and other pretreatments were tested.

Measles infected (or uninfected) alcohol-fixed or unfixed Vero cells were cytospun (800 rpm 4 minutes) onto PLL- or APES-coated slides, air dried and stored at -20°C before use.

The deparaffinised tissue section or cytospin was surrounded by gum and allowed to set (as described previously [figure 1]). An RT mixture was placed within the "well" created by the gum (Cowgum Unit No 1133, Cow Proofings Ltd, Slough, UK), so that the mixture covered the tissue. A plastic coverslip was then placed onto the gum creating a seal (Figure 1). The flat surface (or inserted heating block) of the PCR machine (Techne PHC-2 Thermal Cycler, Techne Ltd, Cambridge, UK) was covered by a heat resistant, rapid temperature transmitting plastic tray (Techne Hi Temp Microplate, Techne Ltd, Cambridge, UK). Subsequently, the tissue sections or cytospins on slides were placed on to this tray (Figure 1). Reverse transcription was performed

as follows: 28°C 10 minutes; 42°C 60 minutes; 95°C 5 minutes; 0°C 5 minutes. After the reaction was completed, the coverslip was removed (Figure 2), the gum scraped off after removing the residual RT mixture covering the tissue for future gel electrophoresis, and the slide air dried prior to storage at -20°C. DNA was also extracted from measles infected and uninfected tissue by standard techniques, and submitted for standard RT/PCR analysis as controls.

The RT mixture used was prepared according to the following specifications (partly using the Perkin Elmer GeneAmp RNA PCR kit, Perkin Elmer Corp, Norwalk, USA) per slide: 10 μl 25 mM $MgCl_2$, 5 μl of 10 x PCR buffer, each dNTP 5 μl 10 mM, 5 μl distilled water, reverse transcriptase (recombinant Moloney Murine leukaemia virus) 5.0 μl (50 units/μl), RNase inhibitor 5.0 μl (20 units/μl), random hexamers 5.0 μl (50 mM solution).

Intracellular PCR on cDNA of measles within tissue sections or cytospins

Tissue sections or cytospins were surrounded by heat-resistant gum (Cowgum Unit No 1133, Cow Proofings Ltd, Slough, UK) and the gum allowed to set. The flat surface (or inserted heating block) of the PCR machine (Techne PHC-2 Thermal Cycler, Techne Ltd, Cambridge, UK) was covered by a heat-resistant, rapid temperature transmitting plastic tray (Techne Hi Temp Microplate, Techne Ltd, Cambridge, UK). Subsequently, the tissue sections on slides were placed onto this tray (Figure 1).

An isotonic PCR mixture was prepared according to the following specifications (partly using the Perkin Elmer GeneAmp RNA PCR kit, Perkin Elmer Corp, Norwalk, USA) per slide: 10 μl 25 mM $MgCl_2$, 20 μl 10 x PCR buffer, 5 μl 10 mM each dNTP, 164 μl dH_2O, 2 μl each primer pair 460 μg/ml,1 μl 5 U/μl Taq polymerase (Amphitaq), 200 μl PBS. The isotonic PCR mixture was then placed onto the slide so that it adequately covered the tissue sample or cellular material of interest, and a heat resistant plastic coverslip was placed onto the gum enclosing the PCR mixture covering the section. After initial Denaturation 94°C for 5-10 minutes; thermal cycling parameters used were: Denaturation: 94°C, 1 minute/Reannealing: 58°C or 63°C, 2 minutes/Extension: 74°C, 1½ minutes with a 5 second extension added to each consecutive cycle, 40 cycles. An extension step after thermal cycling was used 74°C, 5-10 minutes. No vapour barrier (eg mineral oil) was used.

After the thermal cycling was completed, the coverslip was lifted off, the residual PCR mix overlying the tissue section or cells stored for subsequent gel electrophoresis, then the gum was scraped off, and the slide washed in PBS and air-dried. Subsequently, the slide was stored at -20°C for future ISH. When ISH detection was used the slides were submitted to only one round of PCR (ie 30-40 cycles) using only the inner flanking primers MV3 5' AGC ATC TGA ACT CGG TAT CAC 3' and MV4 5' AGC TCT CGC ATC ACT TGC TCT 3'.

In nested *in situ* PCR reactions, for first round PCR outer flanking primers MV1 and MV2 instead of inner flanking primers MV3 and MV4 were used, and the slide preparation for reaction and PCR mixture components were otherwise identical to the above for non-nested PCR reactions. The sequences of MV1 and MV2 were 5' TTA GGG CAA GAG ATG GTA AGG 3' and 5' GTT CTT CCG AGA TTC CTG CCA 3' respectively. After first round thermal cycling was completed, the coverslip was lifted off, the residual PCR mix overlying the tissue section or cells stored for subsequent gel electrophoresis, then the gum was scraped off, and the slide washed in PBS and air-dried. In nested PCR reactions, for second round PCR inner flanking primers (MV3, MV4) were used, and the 5 μl 10 mM dTTP was replaced by 2.5 μl 10 mM dTTP and 2 μl digoxigenin labelled-11-dUTP (25 nmol) (Dig-11-dUTP). Otherwise the slide preparation for reaction and PCR mixture components were identical to that described above. After the second round thermal cycling was completed, the coverslip was lifted off, the residual PCR mix overlying the tissue or cells stored for subsequent gel electrophoresis. Then the gum was scraped off, and the slide washed in PBS and air dried and stored at -20°C prior to detection of digoxigenin incorporated into amplificant material.

ISH detection of measles amplificants using digoxigenin-labelled riboprobe

1. Wash in diethylpyrocarbonate (DEPC) distilled water (5 minutes).
2. Wash in PBS/2 mM Ethylene Diammine Tetracetic Acid (EDTA) (5 minutes).
3. Proteinase K treatment (100-1000 μg/ml) 37°C for 15 minutes in above buffer (PBS/EDTA).
4. Rinse 0.2% glycine/PBS mixture (5 minutes).
5. Wash in PBS (5 minutes).

6. Fix in 4% paraformaldehyde/PBS mixture (2 minutes).
7. PBS rinse.
8. Denature slides in DEPC distilled water for 10 minutes at 95°C; then plunge into ice cold water, dehydrate in alcohol and air dry.
9. Hybridisation at 42°C with digoxigenin-labelled antimeasles riboprobe (1 hour).

The riboprobe was obtained as follows:

A transcription vector (pgem I) containing the 3' sequence of the measles virus N-gene inserted in the Bam H1 site of pGem I was provided by Dr Louise Cosby, Queen's University, Belfast. The riboprobe was synthesised using T7 polymerase promoter by standard techniques. This biotin or digoxigenin-labelled riboprobe has been used extensively by Dr Cosby and ourselves, and is specific for measles virus. It is known to hybridise with all measles virus nucleocapsid sequences contained within the Genebank data system and does not hybridise with the closely related morbillivirus, canine distemper virus.

10. Rinse in 2 x SSC for 20 minutes at 37°C.
11. Rnase A 100 μg/ml 2 x SSC at 37°C for 15 minutes.
12. Rinse in 2 x SSC, then 1 x SSC for 20 minutes.
13. Rinse in Tris buffered saline (TBS).
14. Quench in 10% normal rabbit serum (30 minutes).
15. Incubate in a mouse α digoxin (D8156 Sigma clone D1-22) diluted 1/10,000 TBS (1-1½ hours).
16. 3 washes in TBS (5 minutes each).
17. R α M IgG 1/25 (Dako) and 1/25 normal human serum diluted in TBS.
18. 3 washes in TBS (5 minutes each).
19. Alkaline phosphatase Anti-Alkaline Phosphatase complexes (APAAP) diluted 1/50 TBS (for 30 minutes).
20. 3 washes in TBS (5 minutes each)
21. R α M IgG (10 minutes).
22. 3 washes in TBS (5 minutes each).
23. APAAP diluted 1/50 TBS (for 10 minutes +).
24. 3 washes in TBS (5 minutes each).

25. Rinse in 100 Mm TRIS Ph 9.2, 100 Mm sodium chloride, 50 Mm magnesium chloride.
26. Detect using Sigma fast red (5-30 minutes).
27. Rinse in TBS, tapwater, and counterstain with Crazzi's Haematoxylin. Rinse and mount in glycergel (Dako).

Detection of Digoxigenin-labelled amplificants

<u>According to standard protocols</u>.

1. The slides were rinsed in 1 x (TBS) or a suitable isotonic solution (PBS).
2. Digestion was performed 10 µl/ml proteinase K (Sigma P4914) in PBS/Ethylene diamine tetra-acetic acid for 25 minutes at 37°C (200 µl/section).
3. Wash in PBS.
4. Quench in 1 in 25 normal horse serum diluted 1 in 25 TBS.
5. Add anti-digoxigenin-AP-Fab (Boehringer) fragment diluted 1/200 in TBS for 45 minutes.
6. Wash in TBS - 3 washes for 5 minutes each.
7. Equilibrate in AP2 buffer (50 ml Tris, 50 ml $MgCl_2$ 50 mM, 400 ml of water).
8. Fast red stain. 10 ml distilled water and Tris tablet - Tris (hydroxymethyl), (Sigma F4523) - to 10 ml ddH_2O add 1 Tris tablet dissolve then add 1 Fast red tablet, containing fast red substrate levamisole.
9. Wash in PBS. Counterstain with haematoxylin (Crazzi's).

Controls

Rigorous controls were used in every procedure. For each *in situ* RT or *in situ* PCR reaction, samples with all reaction components but with cells replaced by either cell lysates, or no cellular material at all (PBS added instead) were tested simultaneously and the presence or absence of a band of correct size checked by gel electrophoresis after freeze-thawing the sample several times.

Other controls included measles-infected cellular samples in identical *in situ* RT or *in situ* PCR mixtures as test material but without reverse transcriptase enzyme added for the reverse transcription stage, and no Taq polymerase enzyme or no primers or inappropriate primers added for the PCR stage respectively.

In addition, for the ISH detection, dissimilar probes to the test samples were employed. For the correct probe, known positive and negative control samples were examined simultaneously.

For *in situ* nested PCR with Dig-11-dUTP incorporation into the second round, controls included *in situ* RT with and without Dig-11-dUTP, *in situ* PCR first round with and without Dig-11-dUTP, and *in situ* nested PCR without Dig 11-dUTP incorporation in the second round of PCR.

Sequencing studies

Measles amplificants were sequenced from cell lysates after *in situ* RT followed by *in situ* nested PCR. Protocols for sequencing are well known.

Effect of ddNTP labelled Random Blocking Oligonucleotides on Suppressing Non-Specific PCR products for Measles Sequences Detected by Direct *In Situ* RT/PCR on Slides

With measles infected and uninfected cells on slides digested 1mg/ml Proteinase K for 15 minutes, the effect of internal priming nuclear signal was evaluated by omitting primers. The suppression of this nonspecific signal by addition of 3'-ddNTP labelled random blocking oligonucleotides (15μM) to the reaction mixture was investigated as described in the results.

The effect of addition of 3'- ddNTP labelled random blocking oligonucleotides (15μM) to pretreated (1mg/ml Proteinase K for 15 minutes) mixed measles infected and uninfected sections was also investigated, when direct *in situ* RT/PCR was performed using measles-specific primers. Details of reaction are described in the results.

Effect of ddNTP labelled Specific Blocking Nucleotides on Suppressing Nonspecific PCR Products on Closely Related Sequences using Direct *In Situ* RT/PCR

An oligonucleotide sequence (MV3■) almost identical to primer MV3, but with two base pair alterations compared to the primer MV3 sequence, was constructed. (MV3■ 5'AGC GCC TGA ACT CGG TAT CAC 3'). After *in situ* RT, *in situ* PCR was performed with primer MV4, and either primer MV3 or primer MV3■; the effect of adding 3'-ddNTP labelled primer MV3■ either to the *in situ* PCR reaction using primer MV3 or primer MV3■ was investigated.

Effect of Two Downstream Primers in the *In Situ* Reverse Transcription Step within Cells in Suspension

The MV2 primer was used as a "downstream" primer in the *in situ* reverse transcription step; the simultaneous use of a second "downstream" primer MV4 with or without a ddNTP at the 3' end (specific enhancing primer) was investigated. After the *in situ* reverse transcription step, the PCR step was performed with primers MV3 and MV4. Cells were lysed after reaction and gel electrophoresis performed. If appropriate cDNA was present after the *in situ* RT step with both MV2 and MV4 primers (without ddNTP added at the 3' end), a PCR product of expected size would be seen since a template for *in situ* PCR with primers MV3 and MV4 would be present. If however the reverse transcription step produced a cDNA which did not extend beyond the MV4 primer (due to a ddNTP added to the 3' end), the cDNA produced will not act as a template for *in situ* PCR using primers MV3 and MV4; though in reality some PCR product might be present since some RNA template may by chance have only primer MV2 bound but no ddNTP-labelled MV4 primer.

RESULTS ON MEASLES INFECTED CELLS AND TISSUES

Cellular preservation

Cellular morphology was remarkably well preserved with only slight loss of detail after *in situ* combined RT/nested PCR. Antibody staining revealed expected results.

Gel Electrophoresis

Residual PCR mix overlying tissue or cellular specimens on slides either gave no bands or rarely feint bands of correct size after *in situ* RT/PCR.

Combined in situ RT/in situ nested PCR with Digoxigenin dUTP incorporation in second round in situ nested PCR.

Cytoplasmic signal was obtained when the entire *in situ* RT followed by *in situ* nested PCR with Dig-11-dUTP incorporation in the second round of PCR was performed on measles infected vero cells on PLL-coated glass slides.

Subacute sclerosing panencephalitis formalin-fixed, paraffin-embedded tissues on glass slides submitted for *in situ* RT followed by *in situ* nested PCR with Dig-11-dUTP incorporation in the second round of PCR showed impressive signal, which was both intranuclear and cytoplasmic.

All control samples gave expected results. In particular, no digoxigenin signal was found when *in situ* RT followed by *in situ* nested PCR was performed without adding Dig-11-dUTP in the second round of PCR. Similarly, when *in situ* RT followed by *in situ* single round PCR (outer flanking primers MV1 and MV2) was completed, cells placed in a reaction mix containing Dig-11-dUTP suitable for second round *in situ* nested PCR but not submitted for thermal cycling showed no significant signal.

Combined in situ RT/in situ single round PCR with ISH detection

A strikingly superior signal was obtained after combined *in situ* RT/*in situ* single round PCR followed by ISH using a measles specific riboprobe performed on formalin-fixed or unfixed measles infected vero cells, compared to the signal obtained after *in situ* RT only. Other controls gave expected results.

Sequencing of amplificants after in situ RT followed by in situ nested PCR

After combined *in situ* RT followed by *in situ* nested PCR was performed on alcohol-fixed measles-infected vero cells, the cells were lysed and the amplificants sequenced. The amplificants obtained were specific for measles virus.

Effect of ddNTP labelled Random Blocking Oligonucleotides on Suppressing Non-Specific PCR

Products for Measles Sequences Detected by Direct *In situ* RT/PCR on Slides

When digestion of cells on slides of infected and uninfected cells were increased to 1mg/ml from 10 μg/ml and digestion performed for 15 minutes, a nuclear signal was found in both infected and uninfected cells when specific primers were omitted. This is presumably due to repair and internal priming. This signal was markedly decreased or eliminated in intensity when 3'-ddNTP labelled random blocking oligonucleotides were added to the reaction mixture.

For mixed populations of measles infected and uninfected cellular sections, *in situ* RT/PCR using measles specific primers revealed predominantly nuclear signal in both measles infected and uninfected cells with 1 mg/ml digestion for 15 minutes; in addition, cytoplasmic signal was present within infected cells. The nuclear signal was markedly diminished with the addition of 3'-labelled ddNTP random blocking oligonucleotides, but the cytoplasmic signal was preserved.

Nuclear signal is a nonspecific reaction since almost exclusively cytoplasmic signal for measles is found using *in situ* hybridisation or monoclonal antibody with specificity for measles.

Effect of ddNTP labelled Specific Blocking Oligonucleotides on Suppressing Nonspecific PCR Products on Closely Related Sequences using Direct *In Situ* RT/PCR

When pretreated (10μg/ml Proteinase K for 15 minutes) sections of measles-infected vero cells were examined after direct *in situ* RT/PCR using primer MV4 and either primer MV3 or MV3$^{\blacksquare}$, a cytoplasmic signal identical to that previously described was found. Addition of specific blocking primer 3'ddNTP labelled primer MV3$^{\blacksquare}$, used at identical concentration to other respective primers, did not suppress the specific reaction (primers MV3 and MV4), but markedly reduced the nonspecific reaction (primers MV3$^{\blacksquare}$ and MV4).

Effect of Two Downstream Primers in the *In Situ* Reverse Transcription Step within Cells in Suspension

When MV4 and MV2 primers were used in the RT step, a band on gel electrophoresis of expected size was seen after *in situ* PCR using primers MV3 and MV4. When MV4 primer had a ddNTP at the 3' end (specific enhancing primer), no PCR product was seen after *in situ* PCR using MV3 and MV4 primers.

CLAIMS

1. A method for synthesising a target nucleic acid sequence extracellularly or within cells by replication and/or amplification, which method comprises
   (i) Addition of reaction mixture to the cells or extracellular nucleic acid;
   (ii) Addition of at least one specific oligonucleotide primer;
   (iii) Addition of at least one blocking oligonucleotide primer;
   (iv) Synthesising target nucleic acid sequence.

2. A method for synthesising a target nucleic acid sequence extracellularly or within cells by replication and/or amplification, which method comprises:
   (i) Addition of reaction mixture to the cells or extracellular nucleic acid;
   (ii) Addition of at least one specific oligonucleotide primer;
   (iii) Addition of at least one enhancing oligonucleotide primer;
   (iv) Synthesising target nucleic acid sequence.

3. A method for detecting a target nucleic acid sequence within cells, which method comprises amplifying the target sequence by carrying out a method as claimed in claims 1 and 2; carrying out *in situ* hybridisation employing a labelled probe which hybridises to at least part of the target sequence; and detecting the labelled probe which is hybridised to the amplified target sequence.

4. A method for detecting a target nucleic acid sequence within cells, which method comprises amplifying the target sequence by carrying out a method as claimed in claims 1 and 2 employing a labelled primer, a labelled deoxyribonucleoside triphosphate or a labelled ribonucleotide triphosphate in the amplification mixture; and detecting the labelled primer, the labelled deoxyribonucleoside triphosphate or the labelled ribonucleoside triphosphate present in the amplified target sequence.

5. A method for detecting a target nucleic acid sequence, which method comprises amplifying the target sequence within cells by carrying out a method as claimed

in claims 1 and 2; extracting the amplified target sequence from the cells; carrying out gel electrophoresis optionally followed by Southern or Northern blotting; and detecting the target sequence on the gel or blot.

6. A method for sequencing a target nucleic acid sequence, which method comprises amplifying the target sequence extracellularly or within cells by carrying out a method as claimed in claims 1 and 2; extracting the amplified target sequence from the cells; and sequencing the target sequence.

7. A method according to any one of the preceding claims wherein the transcription and/or amplification mixture may be a polymerase chain reaction (PCR) mixture, a reverse transcription (RT) mixture, a reverse transcription-PCR (RT-PCR) mixture, a self-sustained sequence replication (3SR) mixture, a ligase chain reaction (LCR) mixture, a primed *in situ* extension (PRINS) reaction, or other nucleic acid replication and/or amplification mixture.

8. A method according to any one of the preceding claims wherein the replication and/or amplification mixture is iso-osmotic with the cells.

9. A method according to claims wherein the reaction is performed within suspended cells in solution.

10. A method according to any one of the preceding claims wherein the reaction is performed on a first surface with a section or preparation of cellular material.

11. A method according to any of the preceding claims wherein the first surface is of a heat resistant plastic material.

12. A method according to any one of the preceding claims wherein the first surface is a surface of a microscope slide.

13. A method according to any one of the preceding claims wherein the reaction is performed on extracted nucleic acid material in solution.

14. A method according to any one of the preceding claims wherein the blocking or enhancing oligonucleotides are of random sequences.

15. A method according to claims 1 to 13 wherein the blocking or enhancing oligonucleotides are of known sequences.

16. A method according to any one of the preceding claims wherein the blocking or enhancing oligonucleotides have a dideoxynucleotide at the 3'-end of their respective sequences.

17. A kit for performing a replication reaction on extracellular nucleic acid which kit comprises blocking and/or enhancing oligonucleotides, and at least one reagent necessary for replication of a target sequence.

18. A kit for performing an amplification reaction on extracellular nucleic acid which kit comprises blocking and/or enhancing oligonucleotides, and at least one reagent necessary for amplification of a target sequence.

19. A kit for performing a replication reaction within cells, which kit comprises blocking and/or enhancing oligonucleotides, and at least one reagent necessary for replication of a target sequence.

20. A kit for performing an amplification reaction within cells, which kit comprises blocking and/or enhancing oligonucleotides, and at least one reagent necessary for amplification of a target sequence.

21. A kit according to claims 17 to 20 wherein the blocking oligonucleotides are of known nucleotide sequences.

22. A kit according to claims 17 to 208 wherein the blocking oligonucleotides are of random nucleotide sequences.

23. A kit according to claims 17 to 22 wherein the blocking oligonucleotides have a dideoxynucleotide at the 3'-end of the blocking oligonucleotides.

Patents Act 1977 **Examiner's report to the Comptroller under Section 17** **(The Search report)**	Application number GB 9418438.9
Relevant Technical Fields (i) UK Cl (Ed.M) G1B (BAC) (ii) Int Cl (Ed.5) C12Q 1/68	Search Examiner DR N CURTIS Date of completion of Search 22 NOVEMBER 1994
Databases (see below) (i) UK Patent Office collections of GB, EP, WO and US patent specifications. (ii) ONLINE DATABASES: WPI, BIOTECH (DIALOG)	Documents considered relevant following a search in respect of Claims :- 1, 3 TO 23

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Category	Identity of document and relevant passages	Relevant to claim(s)
	NONE	

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